

6 PPTS

09/284233

PCT/EP

12 APR 1999

WO 98/16552

PCT/EP97/04744

Helicobacter pylori live vaccine

Specification

5 The present invention relates to novel recombinant live vaccines, which provide protective immunity against an infection by *Helicobacter pylori* and a method of screening *H. pylori* antigens for optimized vaccines.

10

Helicobacter is a gram-negative bacterial pathogen associated with the development of gastritis, peptic ulceration and gastric carcinoma. Several *Helicobacter* species colonize the stomach, most notably *H. pylori*, *H. heilmanii* and *H. felis*.

15 Although *H. pylori* is the species most commonly associated with human infection, *H. heilmanii* and *H. felis* also have been found to infect humans. High *H. pylori* infection rates are observed in third world countries, as well as in industrialized countries. Among all the virulence factors described in *H.*
20 *pylori*, urease is known to be essential for colonisation of gnotobiotic pigs and nude mice. Urease is an enzyme composed of two structural subunits (UreA and UreB). Previous studies have indicated that oral immunization using recombinant UreB plus cholera toxin were able to protect mice from gastric colonisa-
25 tion with *H. felis* and *H. pylori* (Michetti et al., Gastroenterology 107 (1994), 1002-1011). By oral administration of recombinant UreB antigens, however, in several cases only an incomplete protection can be obtained. Other *H. pylori* antigens shown to give partial protection are the 87 kD vacuolar
30 cytotoxin VacA (Cover and Blaser, J. Biol. Chem. 267 (1992), 10570; Marchetti et al., Science 267 (1995), 1655) and the 13 and 58 kD heat shock proteins HspA and HspB (Ferrero et al., Proc. Natl. Acad. Sci. USA 92 (1995), 6499).

35 Attenuated pathogens, e.g. bacteria, such as *Salmonella*, are known to be efficient live vaccines. The first indications of the efficacy of attenuated *Salmonella* as good vaccine in hu-

- 2 -

mans came from studies using a chemically mutagenized *Salmonella typhi* Ty21a strain (Germanier and Furer, J. Infect. Dis. 141 (1975), 553-558), tested successfully in adult volunteers (Gilman et al., J. Infect. Dis. 136 (1977), 717-723) and later
5 on in children in a large field trial in Egypt (Whadan et al., J. Infect. Dis. 145 (1982), 292-295). The orally administered Ty21a vaccine was able to protect 96% of the Egyptian children vaccinated during three years of surveillance. Since that time new attenuated *Salmonella* live vector vaccines have developed
10 (Hone et al., Vaccine 9 (1991), 810-816), in which well defined mutations incorporated into the chromosome gave rise to non-virulent strains able to induce strong immune responses after oral administration (Tacket et al., Vaccine 10 (1992), 443-446 and Tacket et al., Infect. Immun. 60 (1992), 536-541).
15 Other advantages of the live attenuated *Salmonella* vaccine include its safety, easy administration, long-time protection and no adverse reactions in comparison with the former inactivated wholesale typhoid vaccines (Levine et al., Typhoid Fever Vaccines. In: Plotkin S.A., Mortimer E.A. Jr. (eds.) Vaccines. Philadelphia: WB Saunders (1988), 333-361).
20

Mutants of *S. typhimurium* have been extensively used to deliver antigens because of the possibility to use mice as an animal model, which is believed to mimic *S. typhi* infections
25 in humans. The attenuation of *S. typhimurium* most commonly used consists in site directed mutagenesis of genes affecting the synthesis of aromatic amino acids. Such strains, designated aro mutants, have a negligible pathogenicity, as demonstrated in animal models and human trials using these constructs
30 (Hoiseth and Stocker, Nature 291 (1981), 238-239; Tacket et al. (1992), Supra). Advantage has been taken from the potent immunogenicity of live *Salmonella* vaccine to deliver heterologous antigens. Expression of specific antigens in attenuated *Salmonella* has conferred murine protection against several
35 bacterial pathogens. The use of recombinant live vaccines, which are capable of expressing *Helicobacter* antigens and protecting the vaccinated animals, has not yet been described.

- 3 -

The use of attenuated live vaccines for the treatment of a Helicobacter infection has also not been rendered obvious. The reason therefor being that in the course of the Helicobacter infection a strong immune response against the pathogen per se is induced, which, however, does not lead to a protective immunity. Thus, it was highly surprising that a protective immune response is achieved when using recombinant attenuated bacterial cells as antigen carriers, which are capable of expressing a DNA molecule encoding a Helicobacter antigen.

10 Apparently, recombinant attenuated bacterial cells expressing a Helicobacter antigen are capable of creating a qualitatively different immune response against the heterologous Helicobacter antigen than Helicobacter itself does against its own homologous antigen. Surprisingly, a non-protective immune

15 response is thus transformed into an immune response protecting against Helicobacter infections. This unexpected observation renders it possible to use recombinant attenuated pathogens, e.g. bacterial cells, particularly Salmonella, as carriers for the screening of protective antigens, to apply the

20 protective antigens identified in this manner in any vaccine against Helicobacter infections, and to use recombinant attenuated bacteria as carriers of protective antigens for the immunization against Helicobacter infections in humans and other mammals.

25 Thus, a subject matter of the present invention is a recombinant attenuated pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable to express said nucleic acid

30 molecule or capable to cause the expression of said nucleic acid in a target cell. Preferably the nucleic acid molecule is a DNA molecule.

The attenuated pathogen is a microorganism strain which is

35 able to cause infection and preferably effective immunological protection against the actual pathogen but is no longer pathogenic per se. The attenuated pathogen can be a bacterium, a

- 4 -

virus, a fungus or a parasite. Preferably it is a bacterium, e.g. *Salmonella*, such as *S. typhimurium* or *S. typhi*, *Vibrio cholerae* (Mekalanos et al., *Nature* 306 (1983), 551-557), *Shigella* Species such as *S. flexneri* (Sizemore et al., *Science* 270 (1995), 299-302; Mounier et al., *EMBO J.* 11 (1992), 1991-1999), *Listeria* such as *L. monocytogenes* (Milon and Cossart, *Trends in Microbiology* 3 (1995), 451-453), *Escherichia coli*, *Streptococcus*, such as *S. gordonii* (Medaglini et al., *Proc. Natl. Acad. Sci. USA* 92 (1995) 6868-6872) or *Mycobacterium*, such as *Bacille Calmette Guerin* (Flynn, *Cell. Mol. Biol.* 40 Suppl. 1 (1994), 31-36). More preferably the pathogen is an attenuated enterobacterium such as *Vibrio cholerae*, *Shigella flexneri*, *Escherichia coli* or *Salmonella*. Most preferably the attenuated pathogen is a *Salmonella* cell, e.g. a *Salmonella* aro mutant cell. The attenuated pathogen, however, can be a virus, e.g. an attenuated vaccinia virus, adenovirus or pox virus.

The nucleic acid molecule which is inserted into the pathogen codes for a *Helicobacter* antigen, preferably a *H. felis*, *H. heilmanii* or *H. pylori* antigen, more preferably a *H. pylori* antigen. The *Helicobacter* antigen can be a native *Helicobacter* polypeptide, an immunologically reactive fragment thereof, or an immunologically reactive variant of a native polypeptide or of a fragment thereof. Further, the *Helicobacter* antigen can be a protective carbohydrate or a peptide mimotope simulating the three-dimensional structure of a native *Helicobacter* antigen. Peptide mimotopes can be obtained from peptide libraries presented on the surface of bacterial cells (cf. PCT/EP96/01130). Of course, the transformed cell can also contain several DNA molecules coding for different *Helicobacter* antigens.

The nucleic acid molecules coding for *Helicobacter* antigens may be located on an extrachromosomal vector, e.g. a plasmid, and/or integrated in the cellular chromosome of the pathogen. When the pathogen is used as a vaccine, chromosomal integra-

- 5 -

tion usually is preferred.

Attenuated bacteria can be used to transcribe and translate said nucleic acid molecule directly in the bacterial cell or
5 to deliver said nucleic acid molecule to the infected target cell, such that the DNA molecule is transcribed and/or translated by the eukaryotic target cell machinery. This indirect bacterial vaccination procedure, termed here as genetic vaccination, has been successfully used with *Shigella* as a carrier
10 (Sizemore, D. R., Branstrom, A. A. & Sadoff, J. C. (1995) Attenuated *Shigella* as a DNA delivery vehicle for DNA-mediated immunization. *Science* 270:299-302).

In a preferred embodiment of the present invention the *Helicobacter* antigen is urease, a urease subunit or an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. In a further preferred embodiment of the present invention the *Helicobacter* antigen is a secretory polypeptide from *Helicobacter*, an immunologically reactive
20 variant or fragment thereof or a peptide mimotope thereof. A process for identifying *Helicobacter* genes coding for such secretory polypeptides, and particularly for adhesins, has been disclosed in the international patent application PCT/EP96/02544, which is incorporated herein by reference.

25 This process comprises

- a) preparing a gene bank of *H. pylori* DNA in a host organism containing an inducible transposon coupled to a marker of secretory activity,
 - b) inducing the insertion of the transposon into the *H.*
30 *pylori* DNA and
 - c) conducting a selection for clones containing a secretory gene by means of the marker, and optionally further
 - d) conducting a retransformation of *H. pylori* by means of the DNA of clones containing genes having secretory activity, wherein isogenic *H. pylori* mutant strains are produced by means of integrating the DNA into the chromosome, and
- 35

- 6 -

- e) conducting a selection detecting adherence-deficient *H. pylori* mutant strains.

Suitable examples of antigens obtainable by the above process
5 are selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive variants or fragments thereof or peptide mimotopes thereof. The nucleic and amino acid sequences of the antigens AlpA and AlpB have been disclosed in the international patent applications PCT/EP96/02545 and PCT/
10 EP96/04124, which are incorporated herein by reference. Further, the nucleic and amino acid sequences of AlpB are shown in SEQ ID NO. 1 and 2, and the nucleic and amino acid sequences of AlpA in SEQ ID NO. 3 and 4.

- 15 It is also conceivable, however, that an intracellular antigen is used which can be presented on the surface, e.g. by autolytic release, and confers immunological protection.

The presentation of the *Helicobacter* antigens in the recombi-
20 nant pathogen according to the invention can be accomplished in different ways. The antigen or the antigens can be synthesized in a constitutive, inducible or phase variable manner in the recombinant pathogen. Concerning the constitutive or inducible synthesis of the *Helicobacter* antigens known expres-
25 sion systems can be referred to, as have been described by Sambrook et al., Molecular Cloning, A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press.

Particularly preferred the antigens are presented in a phase
30 variable expression system. Such a phase variable expression system for the production and presentation of foreign antigens in hybrid live vaccines is disclosed in EP-B-0 565 548, which is herein incorporated by reference. In such a phase variable expression system the nucleic acid molecule encoding the *He-*
35 *licobacter* antigen is under control of an expression signal, which is substantially inactive in the pathogen, and which is capable of being activated by a spontaneous reorganization

- 7 -

caused by a nucleic acid, e.g. DNA reorganization mechanism in the pathogen, e.g. a specific DNA inversion process, a specific DNA deletion process, a specific DNA replication process or a specific slipped-strand-mispairing mechanism.

5

A recombinant cell having a phase variable expression system is capable of forming two subpopulations A and B, wherein the division into said subpopulations occurs by spontaneous reorganization in the recombinant nucleic acid, wherein said subpopulation A is capable of infection and immunologically active per se, while subpopulation B, which is regenerated from subpopulation A, produces at least one heterologous Helicobacter antigen and acts immunologically with respect to said additional antigen.

15

The activation of the expression signal encoding the Helicobacter antigen can be directly accomplished by nucleic acid reorganization or, alternatively, indirectly accomplished by activation of a gene encoding a protein which controls the expression of the gene encoding the Helicobacter antigen. The indirect activation represents a system which allows the production of the Helicobacter antigen via a cascade system, which can be realized e.g. in that the gene directly controlled by DNA reorganization codes for an RNA polymerase which is specific for the promoter preceding the Helicobacter gene, or a gene regulator which in another specific manner induces the expression of the Helicobacter gene. In an especially preferred embodiment of the present invention the expression signal for the gene encoding the Helicobacter antigen is a bacteriophage promoter, e.g. a T3, T7 or SP6 promoter, and the activation of the expression signal is caused by a nucleic acid reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

35 The phase variable expression system can be adjusted to provide a preselected expression level of the Helicobacter antigen. This can be accomplished e.g. by modifying the nucleotide

- 8 -

sequence of the expression signal, which is activated by the nucleic acid reorganization mechanism, and/or by inserting further genetic regulation elements.

5 The Helicobacter antigens can be produced in an intracellular, as well as in an extracellular manner in the pathogen according to the invention. For instance, autotransporter systems such as the IgA-protease system (cf. for instance EP-A-0 254 090) or the E. coli AIDA-1 adhesin system (Benz et al., Mol. Microbiol. 6 (1992), 1539) are suited as extracellular secre-
10 tory system. Other suitable outer membrane transporter systems are the RTX-toxin transporters, e.g. the E. coli hemolysin transport system (Hess et al., Proc. Natl. Acad. Sci. USA 93 (1996), 11458-11463).

15 The pathogen according to the invention can contain a second heterologous nucleic acid, e.g. DNA molecule, which codes for an immunomodulatory polypeptide influencing the immune response quantitatively or qualitatively, apart from the nucleic
20 acid molecule encoding the Helicobacter antigen. Examples of such immunomodulatory polypeptides are immune-stimulating peptides, cytokines like IL-2, IL-6 or IL-12, chemokines, toxins, such as cholera toxin B or adhesins.

25 The present invention also refers to a pharmaceutical composition comprising as an active agent a recombinant attenuated pathogen as described above, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants. Preferably, the composition is a living vaccine. The vaccination
30 routes depend upon the choice of the vaccination vector. The administration may be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters such as the vaccinal vector itself, or the route of administration. Usually the dosage comprises about 10^6 to 10^{12} cells
35 (CFU), preferably about 10^8 to 10^{10} cells (CFU) per vaccination. Administration to a mucosal surface (e.g. ocular, intranasal, oral, gastric, intestinal, rectal, vaginal or urinary tract)

- 9 -

or via the parenteral route (e.g. subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal) might be chosen. A method for the preparation of the living vaccine comprises formulating the attenuated pathogen in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

The pharmaceutical composition may be provided in any suitable form, e.g. a suspension in suitable liquid carrier, such as water or milk, a capsule, a tablet etc. In a preferred embodiment of the present invention the composition is a lyophilized product which is suspended in a liquid carrier prior to use.

Further, the present invention refers to a method for preparing a recombinant attenuated pathogen as defined above, comprising the steps of a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein the recombinant pathogen, e.g. a transformed bacterial cell, is obtained; which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell and b) cultivating said recombinant attenuated pathogen under suitable conditions. If the pathogen is a bacterial cell, the nucleic acid molecule encoding the Helicobacter antigen can be located on an extra-chromosomal plasmid. It is, however, also possible to insert the nucleic acid molecule into the chromosome of the pathogen.

Furthermore, the present invention refers to a method for identifying Helicobacter antigens which raise a protective immune response in a mammalian host, comprising the steps of: a) providing an expression gene bank of Helicobacter in an attenuated pathogen and b) screening the clones of the gene bank for the ability to confer a protective immunity against a Helicobacter infection in a mammalian host. Preferably, this identification process takes place in a phase variable expression system, rendering possible a stable expression of all of

- 10 -

the Helicobacter antigens. Recombinant clones can then be applied as "pools" for the oral immunization of test animals, such as mice. The potential of these clones as protective antigen is then determined via a challenge infection with Helicobacter, e.g. a mouse-adapted H. pylori strain. Thus, there is a possibility of directly selecting optimized H. pylori vaccine antigens.

The invention will be further illustrated by the following figures and sequence listings.

Fig. 1: shows a schematic illustration of the urease expression vector pYZ97, whereon the genes coding for the urease subunits UreA and UreB are located under transcriptional control of the T7 promoter $\phi 10$. There is a ribosomal binding site (RBS) between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (ori), a β -lactamase resistance gene (bla) and 4 T7 terminators in series.

Apart from the expression by the T7 promoter, a constitutive low level expression of the urease A and B subunits can also be brought about via a cryptic promoter, which is located upstream from the T7 promoter, on the plasmid pYZ97.

Fig. 2: shows the nucleotide sequence^{SEQ ID NO: 7} of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Fig. 3: shows a schematic illustration of the T7 RNA polymerase (T7RNAP) expression cassettes pYZ88, pYZ84 and pYZ114, which can be integrated into the chromosomes of bacteria.

- 11 -

In the high-expression cassette pYZ88 the lambda PL promoter is located in inverse orientation, upstream from the T7RNAP gene. A gene for the temperature-sensitive repressor cI 857 (cI) is under control of this promoter. A terminator of the bacteriophage fd (fdT) is situated upstream from the cI gene. The gin gene (Mertens, EMBO J. 3 (1984), 2415-2421) codes for a control enzyme of a DNA reorganization mechanism. A DNA sequence coding for the tRNA Arg is located downstream from the gin gene.

In phase A the PL promoter responsible for the expression of the T7RNAP gene is directed in the direction of the cI857 gene and the gin gene. The consequence of this is that an active repressor is formed at the permissive temperature of 28°C and reduces the transcription from the PL promoter. At a higher temperature the transcription of the PL promoter is increased, since the repressor is inactivated at least partially under such external influences. The temperature-dependent increase in the transcription also causes a corresponding increase in the expression of the following gin gene, which as a control enzyme catalyses the inversion of the PL promoter and the transition in phase B, in which the T7RNAP gene is expressed.

In the high-expression system pYZ88 a further fdT transcription terminator is located between a kanamycin-resistance gene (km) and the promoter of this gene. In this manner, the synthesis of an anti-sense RNA, inversely orientated to the T7RNAP gene, which normally contributes to the reduction of the T7RNAP expression, is reduced. This results in a high expression of the T7RNAP.

In the medium-expression system pYZ84 a transcrip-

- 12 -

tion terminator (fdT) is located between the PL promoter and the start of the T7RNAP gene. In this manner the expression of the T7RNAP mRNA is reduced. Additionally, the anti-sense RNA affects the T7RNAP translation. Therefore, only a medium expression occurs.

In the low-expression system pYZ114 a deletion of 100 bp in PL is additionally introduced (Δ PL). In this manner the activity of the PL promoter is reduced to a high extent, which leads to a lower T7RNAP expression and thus to a reduction of the UreA/B gene expression. In this construct the effect of the cryptic promoter on pYZ97 is already observed.

Fig.4: shows the results of an ELISA for anti-H.pylori antibodies in intestinal fluids of vaccinated mice.

Fig.5: shows the results of an ELISA for anti-H.pylori antibodies in the serum of vaccinated mice.

Fig.6: shows the urease activity in the stomach tissue of vaccinated mice after H.pyroli challenge.

SEQ ID NO. 1 and 2 show the nucleotide sequence of the adhesin gene AlpB from H. pylori and the amino acid sequence of the polypeptide coded therefrom.

SEQ ID NO. 3 and 4 show the nucleotide sequence of the adhesin gene AlpA from H. pylori and the amino acid sequence of the protein coded therefrom.

SEQ ID NO. 5 and 6 show the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Experimental partExample 15 Cloning of the ureA and ureB genes.

The structural genes encoding the urease, ureA and ureB, have been genetically cloned from chromosomal DNA of a clinical specimen P1 (formerly 69A) isolated at the University of Amsterdam and provided by Dr. Jos van Putten. The genes
10 were isolated by a PCR-approach using the primer pair YZ019 (5'-GGAATTCCATATGAACTGACTCCCAAAGAG-3') ^{SEQ ID NO: 8} and RH132 (5'-CTGCAGTCGACTAGAAATGCTAAAGAG-3') ^{SEQ ID NO: 9} for amplification. The sequence of the primers was deduced from GenBank (accession numbers M60398, X57132). The DNA sequence of primer YZ019
15 covered the nucleotides 2659-2679 of the published sequence and further contained a translational regulatory sequence (down stream box; Sprengart, M. L. et al., 1990, Nuc. Acid. Res. 18:1719-1723) and a cleavage site for NdeI. The DNA sequence of primer RH132 covered the nucleotides 5071-5088 of
20 the published sequence and a cleavage site for SalI. The amplification product was 2.4 kbp in size comprising the complete coding region of ureA and ureB genes without the original transcriptional start and termination sequences from the *Helicobacter* chromosome. The purified PCR-fragment was
25 digested with NdeI and SalI and inserted into the corresponding cloning sites of T7 expression plasmid pYZ57 to yield the plasmid pYZ97.

pYZ57 was originally derived from plasmid pT7-7, which was described by Tabor (1990, In Current Protocols in Molecular
30 Biology, 16.2.1-16.2.11. Greene Publishing and Wiley-Interscience, New York). Two terminator fragments were introduced into the pT7-7 backbone at different sites by the following strategy: (1) The tandem T7 terminators. A 2.2 kbp EcoRI/HindIII fragment was excised from pEP12 (Brunschwig &
35 Darzins, 1992, Gene, 111:35-41) and the purified fragment ligated with predigested pBA (Mauer, J. et al., 1997, J. Bacteriol. 179:794-804). The ligation product was digested

- 14 -

with *HindIII* and *ClaI*. The resulting 2.2 kbp *HindIII/ClaI* fragment was subsequently inserted into predigested pT7-7. (2) The T1 terminator. A 230 bp *HpaI/NdeI*-fragment was excised from plasmid pDS3EcoRV (provided by Dr. H. Bujard; ZMBH, Heidelberg). The fragment was then further treated with Klenow to generate blunt ends. The purified *rrnBT1* fragment was inserted into the previous pT7-7 derivative, predigested with *BglIII* and subsequently bluntended by Klenow treatment. Figure 1 describes the completed vector pYZ97 used for the expression of the urease genes coding for urease subunits UreA and UreB in *S. typhimurium*. As indicated in Figure 1, the urease genes can be controlled by the T7 promoter $\phi 10$. The ribosome binding site (RBS) is located between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (*ori*) and a β -lactamase resistance gene (*bla*).

Apart from the expression controlled by the T7 promoter, a constitutive moderate level expression of the urease A and B subunits does occur from a promoter driven by *Salmonella* RNA polymerase. The promoter is located upstream from the T7 promoter, on the plasmid pYZ97. For detailed molecular analysis, the purified *BglIII/HindIII*-fragment of pYZ97 was subcloned into the PCR-Script™ SK(+)kit (Stratagene) and subjected to DNA-sequencing. The sequence data confirmed the various elements in their completeness (see Figure 2 and SEQ ID NO.5 and 6): part of the *ureA* gene, the down-stream box, the RBS, the T7 promoter and the T1 terminator (*rrnBT1*). The sequence analysis also disclosed the region between the T1 terminator region and the T7 promoter where the *Salmonella* RNA polymerase promoter is localised. The sequence data suggests a location of this constitutive promoter between nucleotides 222 - 245 which have been deduced from structural predictions (Lisser & Margalit, 1993, Nuc. Acid. Res. 21:1507-1516).

Example 2

Immunological protection by administration of live vaccine

Materials and Methods

- 15 -

Bacterial strains: *S. typhimurium* SL3261 live vector vaccine strain was used as a recipient for the recombinant *H. pylori* urease plasmid constructs. *S. typhimurium* SL3261 is an *aroA* transposon mutant derived from *S. typhimurium* SL1344 wild type strain. *S. typhimurium* SL3261 is a non-virulent strain that gives protection to mice against infection with wild type *S. typhimurium* after oral administration (Hoiseth and Stocker (1981) Supra). *S. typhimurium* SL3261 and derivatives thereof, which contain the urease expression plasmid pYZ97 (extrachromosomal) and the T7RNAP expression cassettes pYZ88, pYZ84 or pYZ114, respectively (integrated into the chromosome) are indicated in table 1. Luria broth or agar was used for bacterial growth at 28°C. *H. pylori* wild type strain grown at 37°C on serum plates was used for the challenge experiments.

Immunization of mice: Four weeks Balb/c mice purchased from Interfauna (Tuttlingen, Germany) were adapted two weeks in an animal facility before being used for experimentation. 150 µl of blood was taken retroorbitally from all mice to obtain preimmune serum. Retroorbital bleedings were repeated from all immunized mice 1 week and 3 weeks after immunization.

Eight groups of 5 mice including controls were used in this study (table 2). Group A, the naive control group, was not immunized with *Salmonella* neither challenged with wild type *H. pylori*. The rest of the groups were all orally immunized. Group B, a negative control group, did not receive *Salmonella* and was challenged with *H. pylori*. Mice from groups C to G were immunized with *Salmonella* vaccine strains and challenged with *H. pylori*. The last group H received recombinant urease B in combination with cholera toxin and was also challenged.

Prior to immunizations mice were left overnight without solid food and 4 hours without water. 100 µl of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Then mice from group B received 100 µl PBS and mice from groups C to G received 1.0×10^{10} CFU

- 16 -

of Salmonella in a 100 μ l volume. Mice from group H received four times 100 μ l of a mixture of recombinant H. pylori UreaseB plus cholera toxin, one dose every week. After every immunization water and food were returned to the mice.

5

H. pylori challenge: Four weeks after the first oral immunization mice from groups B to H were challenged with H. pylori. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 μ l of 3% sodium bicarbonate were given orally to the mice using a stainless steel catheter tube, followed by an oral dose of 5.0×10^9 CFU/ml of Helicobacter pylori. Water and food were returned to the mice after the challenge.

15 Collection of blood and tissues from mice: Twelve weeks after the first immunization the mice were left overnight without food and subsequently sacrificed for analysis of protection and immune response. The mice were anaesthetized with Metoxyfluorane for terminal cardiac bleeding and prior to sacrifice by cervical dislocation. Under aseptic conditions, spleen and stomach were carefully removed from each mouse and placed on ice in separate sterile containers until further processing. Large and small intestine were obtained for further isolation of the intestinal fluid.

25

Processing of stomach and measurement of urease activity: The degree of H. pylori colonisation in the mouse stomach was measured by the presence of active urease in the tissue. The Jatrox-test (Röhm-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers' directions. Stomach mucosa was exposed and washed with PBS, half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing the substrate for measurement of urease activity. Absorbance at 550 nm was measured after tubes were incubated for 4 hours at room temperature. The rest of the stomach tissue was stored at -20°C for further treatments. The urease activity values obtained from the stomach of naive mice, which

- 17 -

did not undergo immunization or challenge, were used to create a base line to indicate the absence of *H. pylori* infection and therefore protection.

5

Table 1UreA and UreB expressing *S. typhimurium* vaccine strains

Strains	Urease Expression	Source
10 <i>S. typhimurium</i> SL3261	Negative	Hoiseth and Stocker
<i>S. typhimurium</i> SL3262 pYZ97	Constitutive Low	this study
<i>S. typhimurium</i> SL3261::pYZ88pYZ97	High T7-induced expression	this study
15 <i>S. typhimurium</i> SL3261::pYZ84pYZ97	Medium T7-induced expression	this study
<i>S. typhimurium</i> SL3261::pYZ114pYZ97	Low T7-induced expression	this study

Table 2

Mice groups used for immunization

Group	Immunogen	No. of oral immunizations
A	None	0
B	PBS oral immunization	1
C	S. typhimurium S3261	1
D	S. typhimurium S3261 pYZ97	1
E	S. typhimurium S3261::pYZ88pYZ97	1
E	S. typhimurium S3261::pYZ84pYZ97	1
G	S. typhimurium S3261::pYZ114pYZ97	1
H	Urease B plus cholera toxin	4

15 Results:

In the control mice (groups B and C) 100% infection with *H. pylori* was observed. In the mice immunized with recombinant attenuated pathogens (groups D, E, F, G) between 0% and 60% infection (100% to 40% protection) was observed. Immuno-protection did not correlate with humoral anti-UreA and UreB response, suggesting that, in addition to humoral immunity, cellular immunity is critical for protection against *H. pylori* infection. The results indicate that oral immunization of mice with UreA and UreB delivered by *S. typhimurium* attenuated strain is effective to induce high levels of protection against *H. pylori* colonisation.

In the mice immunized with recombinant urease B plus cholera toxin considerably higher levels of urease activity were ob-

- 19 -

served under said experimental conditions than when administering the recombinant attenuated pathogens according to the invention.

5 The results of the urease test have been illustrated in table 3.

- 20 -

Table 3

s

Group	Mouse	$E_{550nm, 4h}$	$E_{4h} - E_{control}$	$E_{corr.} \cdot 3$	Dilution
A	1	0,085	-0,022	-0,066	200 μ l+400 μ l
A	2	0,091	-0,016	-0,048	200 μ l+400 μ l
A	3	0,116	0,009	0,027	200 μ l+400 μ l
A	4	0,099	-0,008	-0,024	200 μ l+400 μ l
A	5	0,101	-0,006	-0,018	200 μ l+400 μ l
Control		0,107	0	0	200 μ l+400 μ l
B	1	0,394	0,292	0,876	200 μ l+400 μ l
B	2	0,464	0,362	1,086	200 μ l+400 μ l
B	3	0,329	0,227	0,681	200 μ l+400 μ l
B	4	0,527	0,425	1,275	200 μ l+400 μ l
B	5	0,462	0,36	1,08	200 μ l+400 μ l
Control		0,102	0	0	200 μ l+400 μ l
C	1	0,248	0,145	0,435	200 μ l+400 μ l
C	2	0,369	0,266	0,798	200 μ l+400 μ l
C	3	0,209	0,106	0,318	200 μ l+400 μ l
C	4	0,219	0,116	0,348	200 μ l+400 μ l
C	5	0,24	0,137	0,411	200 μ l+400 μ l
Control		0,103	0	0	200 μ l+400 μ l
D	1	0,143	0,002	0,004	300 μ l+300 μ l
D	2	0,156	0,015	0,03	300 μ l+300 μ l
D	3	0,142	0,001	0,002	300 μ l+300 μ l
D	4	0,114	-0,027	-0,054	300 μ l+300 μ l
D	5	0,133	-0,008	-0,016	300 μ l+300 μ l
Control		0,141	0	0	300 μ l+300 μ l
E	1	0,127	0,027	0,081	200 μ l+400 μ l
E	2	0,094	-0,006	-0,018	200 μ l+400 μ l
E	3	0,099	-0,001	-0,003	200 μ l+400 μ l
E	4	0,161	0,061	0,183	200 μ l+400 μ l
E	5	0,198	0,098	0,294	200 μ l+400 μ l
Control		0,1	0	0	200 μ l+400 μ l
F	1	0,166	0,025	0,05	300 μ l+300 μ l
F	2	0,145	0,004	0,008	300 μ l+300 μ l
F	3	0,166	0,025	0,05	300 μ l+300 μ l
F	4	0,154	0,013	0,026	300 μ l+300 μ l
F	5	0,301	0,16	0,32	300 μ l+300 μ l
Control		0,141	0	0	300 μ l+300 μ l
G	1	0,084	-0,019	-0,057	200 μ l+400 μ l
G	2	0,087	-0,016	-0,048	200 μ l+400 μ l
G	3	0,269	0,166	0,498	200 μ l+400 μ l
G	4	0,085	-0,018	-0,054	200 μ l+400 μ l
G	5	0,092	-0,011	-0,033	200 μ l+400 μ l
Control		0,103	0	0	200 μ l+400 μ l
H	1	0,638	0,531	1,593	200 μ l+400 μ l
H	2	0,282	0,175	0,525	200 μ l+400 μ l
H	3	0,141	0,034	0,102	200 μ l+400 μ l
H	4	0,135	0,028	0,084	200 μ l+400 μ l
H	5	0,171	0,064	0,192	200 μ l+400 μ l
Control		0,107	0	0	200 μ l+400 μ l

Example 3Construction and molecular analysis of various recombinant *S. typhimurium* strains expressing ureA/ureB subunits.5 Description of the *S. typhimurium* strains used for immunization experiments.

S. typhimurium SL3261(pYZ97) (construct A): *S. typhimurium* SL3261 live vaccine vector strain was used as a recipient for the recombinant urease plasmid construct pYZ97.

10 *S. typhimurium* SL3261::YZ Series (pYZ97) (construct B): These carrier strains are a derivative of *S. typhimurium* SL3261 which has been equipped with the T7 RNA polymerase (T7RNAP) expression cassettes schematically presented in Figure 3. These expression cassettes encode the gene for
15 T7RNAP which is expressed in a 2-phase modus (ON/OFF) as disclosed in a previous invention of Yan et al. ("Two phase system for the production and presentation of foreign antigens in hybrid live vaccines", PCT/EP91/02478). The cassette can be integrated into the chromosome of bacteria and provide the
20 cell in ON-position with optimal amount of T7RNAP for activation of T7RNAP-dependent expression plasmids such as pYZ97.

The principle of the YZ84 cassette is an invertible lambda PL promoter placed on a fragment that is inverted by
25 the phage Mu invertase Gin (Yan & Meyer, 1996, J. Biotechnol. 44:197-201). Dependent on the orientation of the PL promoter either the gin gene (OFF-position) or the T7RNAP gene (ON-position) is expressed. The following regulatory elements have been included in YZ84: (1) The temperature-sensitive cI_{ts}
30 lambda repressor (cI) which represses the PL promoter at 28°C and dissociates at 37°C. (2) The phage fd terminator (fdT) reduces expression of gin gene in order to achieve moderate inversion rates of the PL promoter on the invertible fragment.

The 2-phase expression system enables high expression
35 rates of foreign antigens, such as the urease subunits A and B. It is well known that high expression rates of foreign antigens reduce viability of *Salmonella* carrier thus

- 22 -

diminishing immune response and consequently the protective potential. It was shown that the 2-phase system has a natural competence to improve survival of recombinant *Salmonella* which express large amounts of foreign antigen. In construct B, expression of the *ureA* and *ureB* genes is mainly under the control of the strong T7 promoter resulting in high production of the urease subunits. If the T7RNAP expression cassette is in OFF-position and no T7RNAP is present, the *ureA* and *ureB* genes are constitutively expressed in moderate range by the *Salmonella* promoter.

Analysis of *ureA/B* subunits produced by the various *S. typhimurium* strains used for immunization experiments.

Salmonella constructs A and B were first analyzed by SDS-polyacrylamide gels for expression of UreA and UreB. The recombinant strains were grown at 37°C in liquid Luria Broth supplemented with 100 µg/ml Ampicillin starting from an over night culture. The bacteria were harvested at logarithmic growth phase by centrifugation and the cell pellet was resuspended in 10mM Tris-HCl and 10 mM EDTA, (pH 8.0) and cell-density adjusted to standard $A_{590}=1.0$ in all probes. The bacterial suspension was mixed with the same volume of SDS-sample buffer (Sambrook, J. et al. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and boiled for 5 min. 20 µl of suspension were loaded onto two SDS-10 % polyacrylamide gels; one of the gels was stained with Coomassie blue stain and the other was electroblotted onto a nitrocellulose membrane and further processed for immunoblotting. The nitrocellulose membrane carrying the transferred proteins was blocked for 45 min at room temperature in 10 (v/w)% non-fat milk Tris-buffer-saline (TBS) (TrisHCl 100mM, NaCl 150mM, pH 7.2). After three washes in TBS-0,05 (v/v)% Tween-20, a 1:2000 dilution of rabbit anti-UreB antibody (AK 201) in 5 (w/v)% non-fat milk-TBS was added to the strip and incubated overnight at 4°C. Serum was obtained from rabbit immunised with recombinant urease B subunit purified via affinity chromatography. The membrane was washed three times for 10 min with 0,05 (v/v)%

- 23 -

Tween-20 in PBS, and further incubated in 5 (w/v)% non-fat milk-TBS with goat anti-rabbit IgG antibody horse radish peroxidase conjugate for 45 min at room temperature. After three washes with 0.2 (v/v)% Tween-20 as above, the membrane
5 was developed using the ECL kit (Amersham, Germany) following the recommendations of the suppliers.

Construct A: Proteins of 67 kDa and 30 kDa were observed in the Coomassie stained gel of the whole cell lysate of construct A (*S. typhimurium* strain SL3261(pYZ97)); these sizes
10 correlate very well with those of UreB and UreA, respectively. Such proteins were absent in the control lanes containing the *S. typhimurium* SL3261 strain. Immunoblot analysis of the same protein samples using a rabbit anti-UreB antibody confirmed
15 UreB. Expression of *ureB* from *S. typhimurium* strain SL3261(pYZ97) was also examined at different phases of growth by incubating at 37°C for 2, 6 and 11 hours, respectively. Expression of *ureB* was observed in all phases of growth including in the stationary phase; although, higher expression
20 was observed at early phases of growth. The results obtained with strain SL3261(pYZ97) indicate that UreA and UreB proteins are non-toxic for *Salmonella* and can be expressed at 37°C at any phase of bacterial growth.

Construct B: Similar analysis were performed with
25 construct B. The comparison of both constructs in SDS-PAGE analysis reveals that construct B is the more efficient producer whilst construct A has moderate expression of *ureA* and *ureB*. In the course of bacterial growth of construct B, the expression of *ureA* and *ureB* is constantly high over a
30 longer time period even without antibiotic selection. This confirms the exceptional productivity of construct B in comparison to construct A.

In summary, our data indicate that UreA and UreB from *H. pylori* can be expressed in *S. typhimurium* without causing
35 adverse effects to the bacteria, and are, therefore, suitable for animal protection experiments when delivered by *Salmonella* carriers.

- 24 -

Plasmid-stability

Plasmid stability is essential to assure stable expression of antigens coded by genes which have been cloned into such plasmids.

5 In vitro plasmid stability. The ampicillin resistance marker present on plasmid pYZ97 and absent in the plasmidless *S. typhimurium* strain SL3261 was used as an indicator of plasmid stability. *S. typhimurium* strain SL3261 was grown in LB liquid medium at 28°C for up to 100 generations as described
10 previously (Summers, D. K. and D. J. Sherrat. 1984. Cell. 36:1097-1103). Every ten generations, the number of ampicillin resistant CFU was determined from the total number of colony forming units (CFU) of *Salmonella* by plating equal number of bacterial dilutions on plain LB-agar plates and LB-agar plates
15 supplemented with 100 µg/ml ampicillin.

Plasmid stability in vivo. Plasmid stability in vivo was analyzed by examining total CFU and ampicillin resistant CFU from mice spleen, two and seven days after oral infection of mice with 5.0×10^9 CFU of *S. typhimurium* SL3261(pYZ97). Mice
20 were orally infected with *Salmonella* as described above. Two days and seven days after infection mice were sacrificed under metoxyfluorane anesthesia, and the spleen was removed aseptically for further processing. The spleen was dissected in small pieces in a petri dish, mixed with 1 ml ice-cold
25 ddH₂O, and passed several times through a 18 gauge needle to suspend the spleen cells. The cell suspension was then plated on LB-agar plates with or without 100 µg/ml ampicillin. Plates were incubated at 37°C overnight and colonies counted the next day.

30 Plasmid stability in vivo was analyzed after infecting mice with one oral dose of 5.0×10^9 CFU of *S. typhimurium* SL3261(pYZ97). Mice spleens were taken two and seven days after infection, and plated on LB-agar plates for examination of total CFU and ampicillin resistant CFU. 2.0×10^4 ampicillin
35 resistant CFU were isolated from the spleens after 48 h (Table 4). The CFU counts decreased to 56 at 7 days after immunization, but again, all were ampicillin resistant. The

- 25 -

data indicate that plasmid pYZ97 is stable in *Salmonella* under in vitro and in vivo conditions and is suitable for the evaluation of urease subunits as protective antigens against mouse stomach colonization by *H. pylori*. The low recovery of *Salmonella* strain SL3261 seven days after infection confirms the attenuation of this strain which allows its safe use for delivery of urease into mice.

- 26 -

Table 4

Recovery of *S. typhimurium* SL3261pYZ97 strain from mouse spleens and evaluation of pYZ97 plasmid stability *in vivo*.

Time after infection	Total CFU ^a	Percentage of Amp ^r CFU ^b
2 days	2.0X10 ⁴	100
7 days	56	100

^a Number of CFU of *S. typhimurium* isolated on LB plates without antibiotics from the mouse spleens two and seven days after mice had been orally inoculated with 5.0X10⁹ CFU of *S. typhimurium* strain SL3261(pYZ97).

^b Percentage of ampicillin resistant CFU from the total No. of CFU isolated from mouse spleens.

Table 5

Examination of urease activity and streptomycin resistant *H. pylori* in stomach antrum from mice immunized with UreA and UreB-expressing *Salmonella*.

Mice group	No.	Urease activity ^a	CFU ^b
Naive Control Group	5	0.058 ± 0.004	0 ± 0
PBS Control Group	5	0.427 ± 0.059	2.7X10 ³ ± 1.0X10 ³
SL3261pYZ97 ^c	5	0.057 ± 0.006	62.6 ± 97.3

^a Urease activity is a mean value ± standard deviation.

^b Determination of CFU of the streptomycin resistant *H. pylori* P76 strain was carried out by plating a section of antrum stomach on serum plates supplemented with 200 µg/ml of streptomycin. *H. pylori* were recognized based on colony morphology, urease activity, and light microscopy examination. Values correspond to CFU ± standard deviation.

^c Mice immunized with *S. typhimurium* SL3261(pYZ97) expressing *ureA* and *ureB* from *H. pylori* as described in Materials and Methods.

- 27 -

Example 4

Protection experiments with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model.

Description of the *Helicobacter pylori* strains used for the experiments

Urease-deficient *H. pylori* P11 strain is a derivative of P1, generated by transposon shuttle mutagenesis using the
10 TnMax5 mini-transposon as disclosed in the invention of Haas et al. ("Verfahren zur Identifizierung sekretorischer Gene aus *Helicobacter pylori*"; PCT/EP96/02544). Insertion of TnMax5 has been mapped at the 3'-end of the ureA gene resulting in a defect expression of ureA and ureB due to transcriptional
15 coupling of both genes.

Mouse-adapted *H. pylori* P49 strain was originally established by Dr. J. G. Fox (MIT, Boston, MA) from a feline isolate. *H. pylori* P76 strain is a streptomycin-resistant derivative of P49 generated by homologous recombination with
20 chromosomal DNA from streptomycin-resistant *H. pylori* strain NCTC11637 as described by P. Nedenskov-Sorensen (1990, J. Infect. Dis. 161: 365-366).

All *H. pylori* strains were grown at 37°C in a microaerobic atmosphere (5% O₂, 85% N₂, and 10% CO₂) on serum plates
25 (Odenbreit, S. et al. 1996. J. Bacteriol. 178:6960-6967) supplemented with 200 µg/ml of streptomycin when appropriate.

Prophylactic immunization experiments with mice.

Immunization experiments were carried out to test the ability of UreA and B delivered by *Salmonella* to protect mice
30 from stomach colonization by *H. pylori*. In total, 5 independent immunisation experiments have been performed. Each experiment consisted of 5 groups each with 5 mice: (1) naive control group was mice neither immunized with *Salmonella* nor challenged with wild type *H. pylori* P49 or the streptomycin
35 resistant derivative strain P76; (2) PBS control group was non-immunized mice that received PBS and were challenged

- 28 -

orally with *H. pylori*; (3) *Salmonella* control group was mice immunized with attenuated *S. typhimurium* SL3261 strain alone and challenged with *H. pylori*; and (5) the vaccine group was the mice immunized with appropriate recombinant *S. typhimurium* construct (A + B) expressing UreA and UreB and challenged with *H. pylori*.

Prior to immunizations, mice were left overnight without solid food and 4 hours without water. 100 μ l of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Immediately after stomach neutralization, mice from the PBS control group received 100 μ l PBS, and mice from the *Salmonella* control group and *Salmonella* vaccine group, received 5.0×10^9 CFU of *S. typhimurium* strain SL3261 and the various recombinant constructs, respectively, in a total volume of 100 μ l. Water and food were returned to the mice after immunization.

Four weeks after the oral immunization, mice from the PBS control-, *Salmonella* control- and vaccine-groups were challenged with 1.0×10^9 CFU of *H. pylori*. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 μ l of 3% sodium bicarbonate were given orally to mice using a stainless steel catheter tube, followed by an oral dose of 1.0×10^9 CFU/ml of *H. pylori* strains P49 or P76. Water and food were returned to mice after challenge.

25

Example 5

Immunological analyses of protection experiments with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model

Collection of blood and intestinal fluid from mice for serological analyses.

Antibody responses were evaluated from all mice using serum and intestinal fluid. 150 μ l blood were collected retro-orbitally before immunization and three weeks after immunization, before *Helicobacter* infection. The final bleeding was carried out 11 weeks after *Salmonella*

- 29 -

immunization (6 weeks after challenge infection) by terminal cardiac puncture under metoxyfluorane anesthesia. The small intestines were taken from mice at the end of experiment and processed as described before (Elson, C. O. et al 1984. J. Immunol. Meth. 67:101-108) with minor modifications. Briefly, the content of intestines was removed by passing 2 ml of 50mM EDTA pH 7.5 (Riedel) containing 0,1mg/ml Soybean trypsin inhibitor (Sigma). The volume was adjusted to 5 ml with 0.15 M NaCl. The samples were vortexed vigorously, centrifuged 10 min at 2,500 rpm (Heraeus, Germany), and supernatant supplemented with 50 μ l of 100 mM phenylmethylsulfonylfluoride (PMSF) (Serva) in 95% ethanol, followed by centrifugation at 13,000rpm for 20 min at 4°C (Hermes). Supernatants were supplemented with 50 μ l of 100 mM PMSF and 50 μ l of 2% sodium azide (Merck) and left on ice 15 min before addition of 250 μ l of 7% bovine serum albumine (Biomol). The samples were frozen at -20°C until further use.

Analysis of anti-urease antibodies in mouse sera and intestinal mucosa by ELISA.

Oral immunization with *Salmonella* is known to elicit IgA antibody responses. The IgA response against urease subunits in mice immunized with *S. typhimurium* construct A + B and in control mice was assessed by ELISA. A soluble extract of *H. pylori* P1 and its urease-deficient mutant derivative strain P11 was prepared in phosphate-buffer-saline by sonicating five times with a sonifier (Branson, Danbury, Conn.) at 5 sec intervals (35 % pulses) for 45 sec. This suspension was centrifuged at 13,000 rpm (Heraeus, Germany) for 10 min at 4°C to remove intact cells. The supernatant was used as antigen after determination of the protein content using the BioRad kit. 96-well microtiter plates (Nunc, Germany) were coated with 50 μ l aliquot of 50 μ g/ml of antigen in sodium carbonate-bicarbonate buffer pH 9.6 and incubated overnight at 4°C. The wells were blocked with 1.0 (w/v)% non-fat milk in Tris-buffer-saline (TBS) for 45 min at room temperature and washed three times with TBS-0.05% Tween-20. The assays, which were performed in triplicate, used 50 μ l of serum or gut washing

- 30 -

diluted 1:100 or 1:2 respectively in 0.5 (w/v)% non-fat milk-TBS added to the wells and left overnight at 4°C. The wells were then washed three times with TBS-0.05% Tween 20, and a 1:3000 dilution of a goat anti-mouse IgA horse-radish peroxidase-conjugate (Sigma) was added to all wells and incubated overnight at 4°C. The color reaction was developed by incubation at 37°C for 30 min with an orthophenyldiamine substrate in sodium acetate buffer and hydrogen peroxide. The reaction was stopped with 10 N H₂SO₄ and the A₄₉₂ was determined in an ELISA reader (Digiscan, Asys Hitech GmbH, Austria).

Mucosal antibodies: (Construct A) Intestinal fluid was taken from each sacrificed mouse at the end of the experiment (six weeks after the *H. pylori* challenge) and tested for the presence of anti-urease antibodies by using total cell extracts of *H. pylori* wild type (P1) and urease deficient mutant strains (P11). As shown in Fig. 4, the IgA antibody response against the wild type *H. pylori* extract was around 10-fold higher in immunized mice versus non-immunized or naive mice. The mucosal IgA antibody response against the urease-deficient *H. pylori* mutant was very low in all groups of mice indicating that most of the intestinal IgA antibody response in immunized mice was directed against urease.

Serum antibodies: (Construct A) The levels of serum IgA antibodies against a wild type and an urease-deficient *H. pylori* were examined prior to immunization, 3 weeks after immunization (before challenge) and 10 weeks after immunization (6 weeks after challenge with *H. pylori*). As shown in Fig. 5 panel A, the levels of anti-wild type *H. pylori* antibodies in mice immunized with urease-expressing *S. typhimurium* construct A were ~20-fold higher at three weeks and 34-fold higher ten weeks after immunization with respect to the pre-immune serum. The serum IgA antibody response against the urease-deficient *H. pylori* strain at 3 and 10 weeks was low in all groups of mice including the mice immunized with *Salmonella* construct A (Fig. 5, panel B), indicating that most of the IgA antibody response in immunized mice is directed against the urease subunits. Low serum

- 31 -

antibody responses against wild type *H. pylori* were also observed at ten weeks in non-immunized mice suggesting that the *H. pylori* challenge given three weeks earlier was enough to induce a specific antibody response in these mice. The IgA response to wild type *H. pylori* in mice immunized for three weeks with *S. typhimurium* SL3261 (*Salmonella* control group) increased moderately, which may be explained by the presence of antigens in *Salmonella* that are able to induce cross-reacting antibodies against *H. pylori*. In contrast, the antibody response against the urease-deficient *H. pylori* strain in immunized mice was as low as the antibody response of non-immunized mice (Fig 5, panel B). This result suggests that most of the antibody response observed in immunized mice was against urease. Low antibody response against the urease-negative mutant was observed in the 10 weeks sera from mice given PBS or immunized with *S. typhimurium* SL3261, suggesting that the antibody response observed is due to the specific immune response against the *H. pylori* antigens given to these mice three weeks earlier during challenge. A low antibody response against the urease-deficient *H. pylori* strain was observed at three weeks in mice immunized with *Salmonella* either expressing or not expressing urease, but was absent in the mice given PBS. This confirms the presence of cross-reacting epitopes between proteins from *Salmonella* and *H. pylori*, respectively. (Construct B): The serological analysis of mice immunized with the construct B series achieved similar results indicating that higher production of antigen by recombinant *Salmonella* does not significantly increase antibody response.

30 Analysis of anti-urease antibodies in mouse sera by immunoblotting.

Expression of UreA and UreB from *S. typhimurium* necessary for the induction of mice specific immune response against *H. pylori* was analyzed. Identification of in vivo expression of UreA and UreB was carried out by looking for anti-UreA and anti-UreB antibodies in serum of mice immunized with *Salmonella* construct A and control mice. *H. pylori* whole-cell

- 32 -

antigens were prepared from the wild type *H. pylori* strain P1. Bacteria were recovered from 3 serum plates, resuspended in PBS, and harvested by 10 min centrifugation at 5,000 g. The cell pellet was resuspended in 10mM Tris-HCl and 10 mM EDTA, (pH 8.0) and cell-density adjusted to standard $A_{590}=1.0$ in all probes. The bacterial suspension was mixed with same volume of SDS-sample buffer (Sambrook, 1989) and boiled for 5 min. 20 μ l Pellet were loaded onto a SDS-10% polyacrylamide gel. The proteins were electro-blotted onto a nitrocellulose membrane and cut into strips which were blocked for 45 min at room temperature in 10 (v/w)% non-fat milk Tris-buffer-saline (TBS) (TrisHCl 100mM, NaCl 150mM, pH 7.2). After three washes in TBS-0,05 (v/v)% Tween-20, a 1:80 dilution of mouse serum in 5 (w/v)% non-fat milk-TBS was added to the strips and incubated overnight at 4°C. Sera was obtained from mice non-immunized and immunized with *Salmonella*. After three washes, the strips were incubated with a goat anti-mouse IgG horse-radish peroxidase conjugate (Sigma) diluted 1:3000 in 5 (w/v)% non-fat milk-TBS. The ECL chemi-luminescence detection kit (Amersham, Germany) was used for development of blots according to the supplier's directions.

Serum from immunized and non-immunized mice was obtained 3 weeks after immunization prior to the challenge with *H. pylori* and tested against whole-cell lysates of the wild type *H. pylori* P1 strain expressing UreA and UreB. Proteins of 67 kDa and 30 kDa in size, corresponding to UreB and UreA, respectively, were recognized by serum from immunized mice immunized with construct A. These bands were not observed in strips tested with serum from non-immunized mice or mice immunized with *Salmonella* only, suggesting that urease expressed by the *Salmonella* vaccine strain was able to induce a specific antibody response against both UreA and UreB of a wild type *H. pylori* strain. Similar results were obtained with construct B.

35

Example 6

- 33 -

Determination of *H. pylori* colonisation in mice pretreated with the various recombinant *S. typhimurium* strains expressing ureA/ureB subunits in *H. pylori* mouse model

Processing of stomach and measurement of urease activity.

5 Urease-test: Analysis of protection against stomach colonization by *H. pylori* was performed by testing for urease activity in the antral portion of the mouse stomach. Measurement of urease activity is a very reliable, sensitive and specific method to test for the presence of *H. pylori* 10 infection (NIH consensus development on *Helicobacter pylori* in peptic ulcer disease. 1994. *Helicobacter pylori* in peptic ulcer disease. JAMA. 272:65) and is routinely used in clinical settings (Kawanishi, M., S. et al 1995. J. Gastroenterol. 30:16-20; Kamija, S. et al 1993. Eur. J. Epidemiol. 9:450-452; 15 Conti-Nibali, S. et al 1990. Am. J. Gastroenterol. 85:1573-1575) and in animal research (Gottfried, M. R. et al 1990. Am. J. Gastroenterol. 85:813-818). The Jatrox-test (Röhn-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers directions. Eleven weeks after immunization with 20 *Salmonella*, mice were sacrificed and the stomach was carefully removed under aseptic conditions. The stomach was placed in ice-cold PBS in an sterile container, and the mucosa was exposed by making an incision along the minor curvature with a sterile blade. The stomach was rinsed with PBS to remove food 25 residues and dissected to isolate the antral region from the corpus region. Half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing 500 μ l of the urease substrate from Jatrox-test. The stomach sample was incubated 4 h at room temperature and the absorbance at 30 550 nm (A_{550}) measured. The urease activity values obtained from the stomach of naive mice, which did not undergo immunization or challenge, were used to determine the baseline. The baseline corresponded to the average urease activity value from five naive mice stomachs tested plus two times the 35 standard deviation of this average. Urease activity values higher than the baseline were considered *H. pylori* colonization positive and values below the baseline were

- 34 -

considered *H. pylori* colonization negative.

Cultivation experiment: The left portion of the antral region of stomachs obtained from mice challenged with the streptomycin resistant *H. pylori* strain P76 were plated on serum plates supplemented with 200 µg/ml of streptomycin and incubated under standard conditions. After three days incubation, bacteria were identified as *H. pylori* based on colony morphology, microscopic examination, and urease activity. The number of colony forming units (CFU) of *H. pylori* grown on plates was determined from each mouse stomach sample.

Urease test (Construct A vs. B): Mice immunized with $\sim 5.0 \times 10^9$ CFU of *Salmonella* and challenged with 1.0×10^9 CFU of *H. pylori* strain P49, as well as control mice, were sacrificed under anesthesia and a section of the antral region of the stomach was taken for measurement of urease activity. As shown in Fig. 6, 100% of the mice immunized with UreA and B delivered by *Salmonella* construct A had urease activity below the baseline, indicating the absence of *H. pylori* colonisation. In contrast, 100 % of the non-immunized mice (PBS) and the mice immunized with *S. typhimurium* strain SL3261 alone, had urease activity measurements far above the baseline indicating stomach colonization by *H. pylori*. The naive group of mice, which did not undergo immunization or challenge, was used to set the baseline of urease activity.

Salmonella of the construct B-series had urease activity values above the baseline indicating stomach colonization by *H. pylori* challenge strain. However, the urease activities within this group were lower as in the controls suggesting a partial protection status of mice immunized with the *Salmonella* construct B series (Figure 6). Both *Salmonella* constructs, A and B, mediate similar antibody response but differed in expression of ureA and ureB. We conclude from this that the quantity of expressed urease antigen is relevant to gain optimal protection.

Construct A: To correlate stomach colonization by *H. pylori* with urease activity a new protection experiment was

- 35 -

performed by immunizing mice orally with *Salmonella* construct A and challenging them with the streptomycin resistant *H. pylori* P76 strain. Urease activity values correlated with the number of CFU of *H. pylori* identified. In two of the five mice
5 immunized with urease-expressing *Salmonella*, no *H. pylori* CFU were detected and the average number of CFU in all five immunized mice was only 62. In contrast, the number of CFU in non-immunized mice was 2,737, which corresponds to 44-fold higher colonization. These data indicate that mice immunized
10 with urease-expressing *Salmonella* were able to eliminate or significantly decrease colonizing *H. pylori* from mouse stomachs.